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PLANT BUFFER SYSTEMS IN RELATION TO THE ABSORPTION OF BASES BY PLANTS[1, 2]

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INTRODUCTION

In view of the important storage and other functions of the parenchyma tissues of agricultural plants, it may be granted that it is of paramount importance that these tissues be kept in a healthy condition. The work of many investigators suggests that a certain degree of constancy of the hydrogen-ion concentration of such tissues is an important factor. To assist in maintaining the proper reaction, a system of buffering in the vacuolar sap with respect to hydrogen ion is presumably necessary. This paper deals with the buffer systems involved as reflected in the sap[4] obtained by expression. The special feature of the investigation was the use of plants grown under controlled conditions of solution or sand-culture technique. Aside from some earlier work conducted in this laboratory, very little study has been made of sap obtained from plants grown in definitely controlled nutrient solutions.

During recent years, Small and his associates have reported the results of many studies on the hydrogen-ion concentrations of plant tissues. A monograph by Small[(18)] contains the data obtained in his

[1] Received for publication February 20, 1932.

[2] In connection with a general investigation, the first part of which is now reported, it is desired to acknowledge the assistance of a grant received from the American Potash and Chemical Company.

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[4] Various terms are used to designate the fluids expressed from plant tissues: sap, tissue fluids, plant juice, etc. Objections can be made to any term employed. In this paper the common term "sap" is used for convenience.

laboratory, by himself and his coworkers, Martin, Ingold, and Armstrong. Much work has been done on individual tissues, the pH values being obtained colorimetrically by what is termed the "range indicator method" or "R.I.M." A discussion of this work is beyond the scope of this paper. In the consideration of buffer systems, Small and his associates have confined their attention especially to a limited range of pH between 4 and 7. This is doubtless because the pH of the sap of most agricultural plants lies within this range and because changes around the actual pH of the plant may be assumed to be the only ones of importance from the point of view of the preservation of a suitable hydrogen-ion concentration.

In undertaking the work to be presented herein, it was felt that only by a study of the complete buffer system could the buffering mechanism at any point be fully elucidated. The evidence obtained indicates that this supposition was correct. Furthermore, the titration of expressed plant saps to low and high pH values has a distinct interest, apart from the question of pH or of buffering. Reflections are thus obtained of variations in concentration of important metabolic constituents of the plant. The primary purpose of this paper is to present evidence on the relation of plants to their nutrient medium, as reflected by the composition and buffer of the sap expressed from the tissues with a given technique.

TECHNIQUE

The following investigations have all been conducted on the expressed sap of plants. While the contents of the cells of many tissues are represented in the composite sap so obtained, it is certain that the bulk of it comes from parenchymatous tissue. Although it is unquestionably necessary to emphasize and to make allowance for the uncertain and composite character of saps expressed from plant tissues, the important point now is that consistent reflections of metabolic conditions may be obtained by the study of expressed saps under suitably controlled conditions. The ideal of quantitative study of each type of cell or tissue is not yet attainable. Small[18] objects that the pH values of expressed saps are of limited validity owing to the loss of CO_2 when the sap comes in contact with air. Further discussion will try to show that this objection is not so important as it might appear at first sight, at least for the objectives of the present investigation.

The principal methods of obtaining sap from plant tissues are: (1) grinding the material to a pulp and extracting by pressure; (2) injur-

ing the cells with organic subtsances, such as ether, and then obtaining the sap by pressure; (3) freezing, thawing, and pressing plants.

The first method gives juice containing suspended matter, and is hard to filter. The second and third were compared by Copeland[1] of this laboratory, who found that the sap obtained by these methods was similar in character.

The method of freezing and thawing was adopted in the present studies. The plant tissues were placed in closed bottles as soon as harvested, and then immediately set in a freezing chamber, kept at about –15° C. The tissues were later thawed at room temperature, and pressure was applied while the material was still cold.

The screw press consists of a heavy steel casing, into which is first inserted a short cylinder perforated with small holes. The material to be pressed is enclosed in cloth and placed above this cylinder. Another close-fitting steel cylinder about 4 inches in diameter and 8 inches in height is then set in place. Pressure is applied by a shaft threaded through a steel bridge. This shaft is fitted to a 16-inch diameter wheel, which is turned by hand. The sap runs through the lower perforated cylinder and comes out through an opening on the side of the casing. In most cases it is filtered rapidly through filter paper and titrated at once. In some of the earlier experiments it was allowed to stand overnight in a cool place. For present purposes, the use of extremely high pressures was considered unnecessary, and in fact, undesirable. The intention was to secure a sap as nearly as possible approximating the vacuolar sap. The complete disintegration of tissue was not sought.

All measurements were made with the Bunker type of hydrogen electrode. At first the electrode was platinized for 10 seconds after each titration and hydrogen gas was secured by electrolysis of NaOH solution. Later it was found unnecessary to replatinize the electrode so frequently, provided it was dipped in dilute acid and then thoroughly washed with water after each alkali titration. Recently it became more convenient to use hydrogen gas from a cylinder. In both cases the hydrogen gas was passed over heated platinum black in order to remove oxygen. Measurements were made on a Leeds and Northrup potentiometer reading pH directly. The instrument is guaranteed to be accurate to 0.01 pH. More accurate instruments are sometimes employed in experiments on plants, but the inherent biological errors make it very doubtful whether anything is to be gained by further refinement of the physical-chemical technique. The temperature at which all measurements were made was approximately 25° C.

Some experiments were conducted to determine the accuracy of the technique used. Readings of pH taken on a buffer solution of KH_2PO_4 showed a maximum deviation of 0.005 pH from the mean, and readings were recorded to nearest 0.01 pH.

Five 50-gram samples of the tops of wheat plants were taken from a large lot and pressed out separately after freezing. The values showed maximum deviations of 0.03 pH on either side of the mean. As a result of this experiment, differences of pH are not ordinarily considered significant if less than 0.1 pH.

The pressure needed to obtain a representative sample of sap was determined. The first half of the sap could be secured with very little pressure—the second half required the full power of the press. Titration showed that practically identical pH values and buffers were obtained in each half.

The importance of freezing before pressing was shown by the fact that while 5 cc of sap expressed from unfrozen wheat plants required only 2.66 cc of acid and alkali for the buffer over the range of pH 2.0 to 10.5, the same amount of sap from the frozen plants required 6.40 cc to cover the same range. There was also a difference of 0.3 in the initial pH of the sap.

In one experiment, a lot of wheat plants was divided into five portions which were separately frozen and thawed, then successively pressed; pH determinations were made immediately after pressing. The readings were as follows:

1st sample	pH 5.87
2nd sample	pH 5.89
3rd sample	pH 5.92
4th sample	pH 5.91
5th sample	pH 5.93

There appears to have been a rise in pH on standing before pressing, but of less than 0.1 pH. At the same time the effect of standing after pressing was specifically investigated. The pH of a sample of fresh sap was found to be 5.83, and after standing overnight in a cool place it was 5.77. Similar comparisons were made on other samples. The changes were observed to be within the 0.1 pH limit of error noted above.

An experiment was then planned to determine more definitely whether allowing the tissues to stand at room temperature after thawing, but before pressing, had any effect. It was also planned to determine whether standing after cutting, but before freezing, had any effect on the pH of the sap. A collection of buckwheat leaves was made and divided into five portions. Four of these were frozen immediately and

the other was left standing in a closed container in the greenhouse for three hours before freezing. The results are given in table 1.

TABLE 1

EFFECT OF ALLOWING LEAVES TO STAND AFTER HARVESTING BUT BEFORE FREEZING, AND AFTER THAWING BUT BEFORE PRESSING, AND OF SHAKING ELECTRODE WHILE MAKING READING, ON THE pH VALUE OF THE SAP

Sample No.	Treatment	pH value of the sap	
		Without shaking electrode	Shaking electrode while making reading
1	Stood 3 hours before freezing; pressed as soon as thawed	5.35	5.35
2	Frozen at once; pressed as soon as thawed	5.34	5.34
3	Frozen at once; pressed 20 minutes after thawing	5.46	5.46
4	Frozen at once; pressed 3 hours after thawing	5.52	5.45
5	Frozen at once; pressed 3 hours after thawing	5.52	5.45

In the case of sap from plants which stood after thawing, a lower pH value was obtained when the solution was shaken while the electrometric reading was being made. This suggested that some substance was reduced by H_2 at the surface of the electrodes, possibly with the formation of ammonia. Such an effect was not observed with sap from samples pressed immediately after thawing.

In the absence of experimental data any explanation of these observations is speculative. Nightingale, Schermerhorn, and Robbins[17] report an increase in the amino acid content of sweet-potato roots when these were allowed to stand subsequent to thawing. Lincoln and Mulay[11] found that after 24 hours' standing, hydrolysis of proteins had occurred in the bark of pear trees. It is possible that changes of this character might be of greater magnitude in the leaves of plants, and that they are responsible for the observed slight alterations of pH.

After a number of samples of sap have been prepared for titration, it has been the practice to titrate all with alkali and then later all with acid. The acid titration is usually done about 2 hours after the alkali titration. It has been observed that frequently the initial pH taken on the second sample of sap (used for the acid titration) is slightly lower than that of the first. The number of cases in which this has occurred is so great as to make it evident that on standing the H–ion concentration of the sap is often increased. The change is usually less than 0.1 pH, and is probably a result of the formation of organic acids. Considerably longer standing does not seem to produce a further measurable change. Another possible source of error is the condensation of moisture on the cold tissues before pressing. However, such dilution would not appre-

ciably alter the trend of the buffer curves and probably would not change the initial pH beyond limits of other errors. In any given experiment, all sets of plants were treated alike as far as was possible.

THE PLANT BUFFERS

There are many substances which might be responsible for the buffering effect in plant sap. The evidence concerning the more important ones will be given consideration. A survey of the literature suggests the following substances:

1. Soluble protein material
2. Carbonates
3. Phosphates
4. Salts of organic acids
5. Amino acids and their amides

Soluble Protein.—The amphoteric nature of protein material around its isoelectric point suggests that it may be of importance in the living plant, in the maintenance of a definite hydrogen-ion concentration. The experiments of Hurd-Karrer,[4] Martin,[13] and Youden and Denny[21] indicate that actually proteins are not of importance in the buffer system of the sap.

Carbonates.—Carbonates are of importance in the buffer metabolism of blood, and must be considered as possible constituents of plant buffers. Copeland,[1] working in this laboratory, was unable to detect appreciable amounts of carbonates in the sap expressed from young pea plants. Martin[14] reports CO_2 present in sunflower sap, but in amounts probably too small to constitute an important part of the buffer system. Leuthardt[9] considers the amount of carbonates in fruits and succulent plants to be unimportant.

Small[18] lays considerable stress on the CO_2 found in sap. He objects to the use of hydrogen electrode determinations on expressed sap on this basis. He maintains, correctly enough, that when the expressed sap comes in contact with the air, any excess CO_2 will be lost. Furthermore, in the act of saturating the solution with hydrogen, the remaining CO_2 will be lost. However, it is very doubtful whether much CO_2 will be found in the sap from leaves or stems of agricultural plants with the technique usually employed. The plants are generally harvested during a period of illumination when the CO_2 available is being used in photosynthesis. Martin[14] reports 7 per cent CO_2 in the broad bean (*Vicia faba*), but most plants are buffered strongly enough so that the shift in pH caused by such a concentration would be very small.

It may be suggested, therefore, that when the tops of plants are harvested as in the present investigation, CO_2 is of minor importance, either as a determinant of the buffer system or of the actual pH of the sap, at least of the composite sap. The effect of CO_2 in certain specialized cells may fail to be reflected in such sap. Moreover, much of the value of the experiments to be described herein is found in the comparisons of saps obtained by a standard technique, when plants are grown under diverse and known cultural conditions.

Phosphates.—Phosphates have an important rôle in the buffer system of the blood. In addition to buffering between pH 4.5 and 7.0, they have a buffer action around pH 2 and pH 13.

The amounts of phosphate usually found in plant saps are fairly small but around the neutral point may be very important. Martin[12, 13] found that between pH 6 and 7, the portion of the buffer ascribed to phosphate varied from 100 per cent in the case of sunflower, to 50 per cent in broad bean. According to Ingold,[7] phosphates account for only 35 per cent of the buffer between pH 6 and 7 in the potato tuber. It is certain that if phosphates are present, they will have some buffer action. The varying degrees of importance to be assigned to phosphate as a buffer will depend on the amount present, and on the presence or absence of some other substance effective over the same range of pH.

Salts of Organic Acids.—Organic-acid radicals are well-known constituents of plant sap, and earlier, as well as recent investigations, make it evident that such acids as citric, malic, oxalic, tartaric, etc., together with their salts, are of great importance in the plant buffer system.

Amino Acids and Amides.—These substances have received very little attention as being of possible importance in the buffer system. Ingold[7] found that a 3 per cent solution of asparagine had very little buffer between pH 6 and 7. Youden and Denny[21] used a solution of glycocol, comparable with the amino acid nitrogen present in potato extract, and observed very little buffer. Leuthardt[9] believed that glutaminic acid is responsible for the buffer of mesembryanthemenum on the alkaline side of neutrality, but that amides are not important around the actual pH of plant saps. Vickery[19] has found appreciable quantities of aspartic and glutaminic acids in alfalfa sap. Asparagine is well known to occur in many plants.

Other Substances.—Sugars exhibit a buffer effect above pH 9, but a fairly concentrated solution is necessary. While such concentrations are present in some fruits, they are not usually found in the green tissues of agricultural plants.

DUPLICATION OF BUFFER CURVES WITH ARTIFICIAL SOLUTIONS

In 1929,[2] the writer attempted to duplicate the buffer curves of saps from buckwheat stems by an artificial mixture. A resumé of the work (unpublished) will be given here.

It was realized that if proteins were important the system would be extremely complex. Experiments were conducted to see if the proteins could be eliminated from consideration. Boiled and filtered sap was compared by means of buffer titrations with fresh sap, and the curves were found to be identical. In another case wheat plants were divided into two lots, the one being dried and ground, and the other frozen. Water was added to the dried and ground sample to give the same water content as fresh plants, and an extract obtained by the use of pressure. The acid buffer curve of this extract was compared with that of the expressed sap of the frozen plants, and they were found to agree almost exactly.

To separate proteins, the expressed sap of fresh tissues was dialyzed for 60 hours and then titrated. The dialyzed sap had a lower pH and a little more buffer than the fresh sap; but no attempt had been made to inhibit enzyme action, and it is believed that this was responsible for the change in pH. A sample of undialyzed sap which stood for 60 hours had the same pH value as the dialyzed sample. It is fairly certain that the buffering substances can be dialyzed and that proteins are of no great importance, as far as the sap itself is concerned. Obviously the buffering system within the protoplasm cannot be disclosed by experiments of the type reported in this paper.

An attempt was then made to duplicate buffer curves of the expressed sap from the stems of two sets of buckwheat plants. These plants had been grown under controlled culture solution conditions, one solution being fairly high in K and the other low. Slight differences in the curves reflecting the two treatments were noted (fig. 1). An attempt was first made to duplicate the high-K curve, since this was considered to be representative of normal buckwheat stems. A complete inorganic analysis of the sap had been made, and phosphate was used in the concentration determined. By mixing the phosphate with suitable organic acids, the curve for the sap could be duplicated on the acid side of pH 7, but in order to obtain a buffer on the alkaline side, the addition of amino acid and amide was found necessary. The inorganic analysis had shown that most of the cation content was made up by K and most of the

inorganic anion content by NO_3. The total equivalents of all cations and of all inorganic anions, except phosphate, were then calculated and equivalent amounts of KOH and HNO_3 added. The following mixture was found to give a curve approximating fairly closely the high-K curve:

Asparagine	0.012 M
Aspartic acid	.021 M
Malic acid	.020 M
Phosphoric acid	.015 M
KOH	.160 N
HNO_3	0.100 N

The initial pH of the mixture was 4.32.

An attempt was then made to duplicate the low-K curve using different quantities of the same constituents. The amide and amino acid were increased to give the increased alkaline buffer observed. KOH, HNO_3, and phosphate were added in accordance with the indications of the sap analysis. As the initial pH of both high-K and low-K saps had been the same, it was found necessary to lower the organic-acid content to bring the solution to the pH of the original sap. The constituents of the solution were as follows:

Asparagine	0.016 M
Aspartic acid	.026 M
Malic acid	.004 M
Phosphoric acid	.015 M
KOH	.140 N
HNO_3	0.100 N

The initial pH of the solution was 4.22. The buffer curves obtained from the high and low-K mixtures were then compared with the original curves and with each other. They are shown in figure 1.

It was evident that insufficient buffer had been obtained on the acid side of pH 7 in the low-K artificial mixture (fig. 1*D*). It is possible that this could have been corrected by a partial replacement of malic acid by citric acid, which buffers at the required pH. However, the work was suspended at this point, since its purpose was to indicate the classes of substances responsible for the buffer of the sap, rather than the actual substances. It was shown that amino acids and their amides, which had previously not been considered of importance, might play a large part in the buffering effect over the range studied.

Similar results have recently been published by Hurd–Karrer,[5] using mixtures of phosphate, asparagine, leucin, malate, and glucose. She was able to duplicate the buffer curves obtained for the sap of wheat seedlings. The amount of glucose used was about three times as much

as was actually found in the sap. Hurd-Karrer considered that the excess may represent other soluble carbohydrates or other substances buffering above pH 9.5.

As has been emphasized by Hurd-Karrer[13] and by the writer,[2] it is possible that the same buffer curves could be obtained using an entirely different group of substances. That the curves could be duplicated by other substances known to occur in plant sap is not so likely.

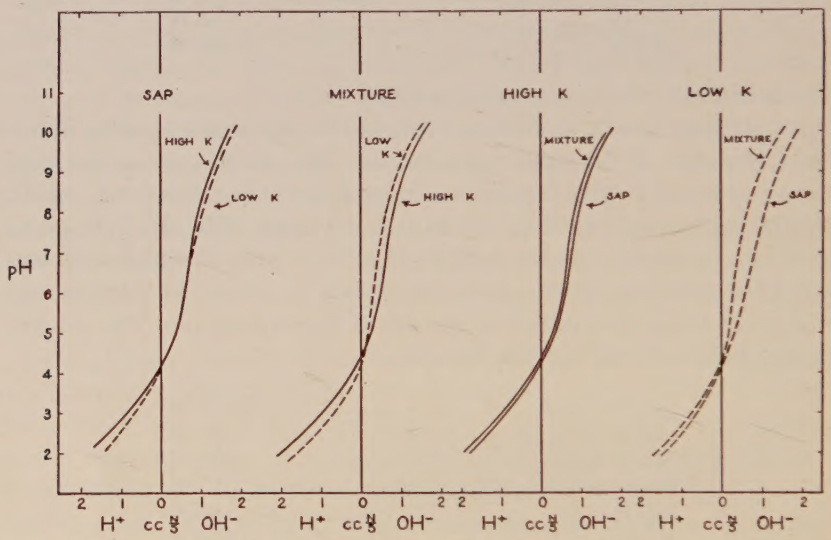

Fig. 1. Comparison of buffer curves of artificial mixtures containing different amounts of K, with each other and with curves of buckwheat stem sap, high and low in K; 5 cc sap was used in titration.

As Hurd-Karrer has pointed out, "it does not seem probable that the close agreement between the titration values of the buffer mixtures and those of the different juice samples is entirely fortuitous." In spite of this, a complete analysis of the sap is necessary before anything can be regarded as proved.

It seems reasonably certain that the buffer on the acid side is mainly due to organic-acid radicals. The alkaline side is more problematical. Analyses of buckwheat sap were therefore made to determine whether aspartic acid and asparagine, when substituted mol for mol for the amino acid nitrogen and the amide nitrogen found in the sap, would duplicate the buffer on the alkaline side of neutrality. The amino nitrogen was determined by use of the Van Slyke apparatus. For amide nitrogen the sap was digested with HCl, made alkaline with MgO, and the ammonia distilled over into H_2SO_4. Recent experiments by Vickery and

Pucher[20] indicate that H_2SO_4 rather than HCl should be used in hydrolysis for amides, otherwise low values may be obtained. The amino nitrogen was found to be 0.0077 M and the amide nitrogen to be 0.004 M. The methods available for determining the organic acids were unsatisfactory, but an estimate of 0.03 M was made for malic acid. Phosphate was found to be low, only 100 p.p.m. being present. For purposes of titration, this was regarded as 0.001 M.

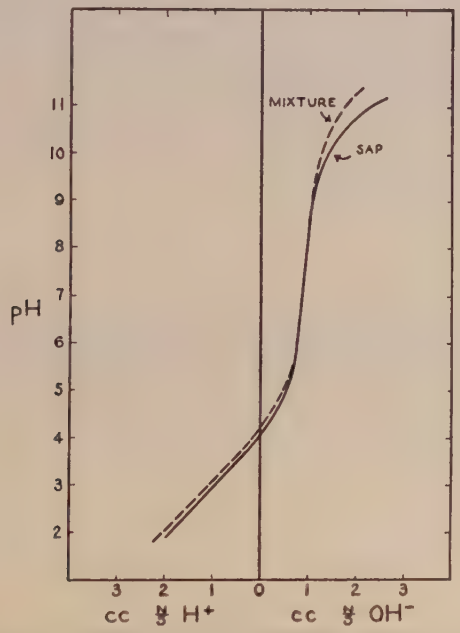

Fig. 2. Comparison of buffer curves of artificial mixture and of sap from buckwheat stems; 5 cc sap was used in titration.

These substances were then mixed to the above concentrations and a titration curve made. This was compared with the curve of the original buckwheat sap. There was not enough buffer above pH 9. Glucose was therefore added to give $\frac{M}{2}$ concentration, but even then the original curve was not duplicated. While fairly close agreement (fig. 2) was obtained on the acid side, the curves separated above pH 10. The artificial mixture used was as follows:

Aspartic acid	0.008 M
Asparagine	.004 M
Malic acid	.03 M
Oxalic acid	.01 M
Phosphoric acid	.001 M
Glucose	0.5 M (7 per cent)

To this mixture NaOH was added to bring it to the pH of the original sap. The mixture was then titrated with HCl and NaOH as in the case of the sap.

The results clearly show a discrepancy on the alkaline side, particularly as it is extremely unlikely that 7 per cent glucose would be found in buckwheat sap. However, if leucin had been used as by Hurd-Karrer, or perhaps a mixture of leucin and aspartic acid, better agreement might have been obtained. The alkaline pK value for aspartic acid is 12.1, while for leucin it is 9.8. This latter should provide more buffer over the pH range where the discrepancy is most marked. The asparagine added seems to supply enough buffer over the part of the curve represented by that substance.[5]

This experiment suggests further that the classes of substances indicated independently by Hurd-Karrer and by the writer as being responsible for the buffering effect in many types of agricultural plants are probably the correct ones.

While the evidence presented earlier indicates that the buffer effect of one type of substance will merge into that of another type, the buffering range of each is sufficiently definite to give some information concerning changes which have taken place. At least, if two sets of plants grown under different conditions should give the same pH value and identical buffer curves over a sufficient range, it is likely that the principal organic constituents of the sap would not differ to any great extent. An attempt was made to estimate approximately the amino acid content of a sap from titration data, but owing to the overlapping of curves for amides, sugars, and amino acids, this was found to be impractical. However, it is believed that a qualitative idea of the concentrations of amino acids, amides, and total organic acids can be obtained by inspection of buffer curves. Small[18] has shown that it is often enlightening to compute "buffer indices" from the titration curves. In connection with the present discussion, it has not seemed essential to present the data in this form, since the titration curves clearly show the influence of the culture media on the buffer system of the sap. The general classes of substances involved in the buffer system are indicated by inspection of the curves and from other data. The amount of sap used in all titrations was 5 cc, and from the data presented, "buffer indices" can be computed if desired.

[5] It has been suggested that phenols and catechols may possibly have a buffer effect in the alkaline range.

INFLUENCE OF ILLUMINATION OF PLANT ON pH OF SAP

Diurnal changes in the pH of sap from succulent plants are known to occur. Such changes are often of large magnitude. The evidence for similar variations in agricultural plants has been recently reviewed by Loehwing.[10] Perhaps the largest changes are those cited by Ingalls and Shive.[6] They report that buckwheat stem sap may vary from pH 4.4 to 4.8 and leaf sap from pH 4.9 to 5.4, according to the time of day. Accumulation of organic acids during the night and photolysis during the day is believed to be responsible for these changes. Such large differences had not been observed in this investigation. An attempt was therefore made to accentuate the effects of light and darkness.

Buckwheat plants growing in a culture solution were selected and divided into three sets. Set 1 was harvested in the afternoon and at the same time set 2 was placed in darkness. On the following day, set 2 was harvested after 24 hours in darkness, and at the same time set 3, which had been in light all day, was also cut. These tissues were all frozen immediately after harvesting and subsequently used for pH determinations.

The buckwheat plants were pressed out immediately on thawing, with the following results:

	pH
Set 1	5.28
Set 2	5.21
Set 3	5.27

In a similar experiment with tomato plants which were let stand some time at room temperature before pressing, the following values were obtained:

	pH of stem	pH of leaves
Set 1	5.66	5.73
Set 2	5.61	5.65
Set 3	5.68	5.80

The changes of pH are very small and may not be significant.

It must also be concluded, from the work of Loehwing[10] that while changes in pH may be produced by the effect of light, they are not always of the large magnitude reported by Ingalls and Shive. Data thus far published do not permit comparisons of temperature effects during the dark periods. It is to be kept in mind that in the present investigation, plants from any one experiment were harvested as nearly as possible at the same time of day.

A few experiments were made to ascertain whether the pH of the expressed sap could be altered by treating the plants with the rays from a mercury arc lamp (5 minutes' daily exposure for 2 weeks). No significant change in pH was found in these particular experiments.

INORGANIC NUTRITION OF THE PLANT IN RELATION TO BUFFER SYSTEMS

Apart from the effect of calcium, little investigation has been made of changes in the buffer system of the sap induced by modifications in the inorganic nutrition of the plant. It is, of course, logical to endeavor to explain the buffer components before attempting to identify variations in the curves. Some of the work reported below was done in the hope that it might throw some further light on the constituents of the buffer system. In the main, however, it was carried out when some knowledge of the system had already been obtained, as described in the preceding sections of the paper.

Phosphorus.—Martin[14] has shown that in bean plants, over a narrow range of pH, the concentration of phosphate in the plant sap may account for all the buffer. That this is not always the case was demonstrated by Ingold,[7] who found that in potato tuber the phosphate could account for only about 30 per cent of the buffer over the same range of pH.

In view of the marked effects of subjecting plants to a low supply of phosphate, it was considered probable that the cell sap might show some change as a result of the evidently deranged metabolism. Water-culture experiments with wheat plants were carried out. The phosphate supply in the low-phosphate set was decreased enough to produce a marked decrease of yield. Except for decreased yield, these plants appeared normal. The sap was obtained and titrated.

It is evident that there was a small decrease of pH resulting from the low phosphate supply. There was also a considerable increase in the buffer on the alkaline side, which probably indicates an increase in amides, amino acids, or sugars (fig. 3). Kraybill[8] has reported analytical data showing an increase of amide and amino nitrogen in plants grown under conditions of low phosphate supply, and similar results have been obtained in this laboratory.

Calcium.—Various earlier investigations have emphasized the assumed necessity for Ca or $CaCO_3$ for neutralization of organic acids produced in the course of plant metabolism. The effects of liming soils

on the reaction of plant sap have received much attention. Frequently the results of such experiments are lacking in consistency, and the biological errors involved have not always been given due consideration.

Loehwing[10] has recently studied wheat plants grown on humus and loam soils. The plants from the lime-treated soils in all cases showed a decrease in acidity. There was a larger change in pH in the plants from the humus soil treated with lime than in those from the loam soil,

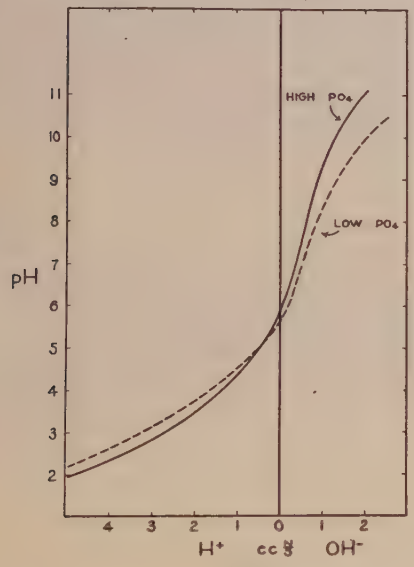

Fig. 3

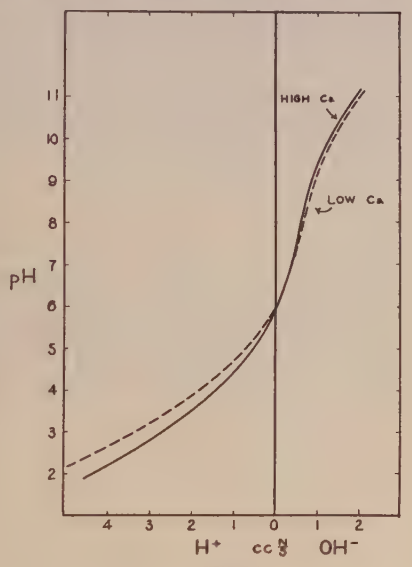

Fig. 4

Figs. 3 and 4. Buffer curves for tops of wheat plants (Little Club) grown in solutions indicated in each chart; 5 cc of sap was used for titration.

The solutions were of the type described in table 4. In the low-PO_4 solution, KH_2PO_4 was used in 0.0001 M concentration.

The plants were grown in a greenhouse approximately 6 weeks from February 24, 1928. Two-liter jars were employed, with two plants in each jar.

but the former showed signs of chlorosis. Loehwing considered that the acidity developed in plants grown on the untreated humus soil and the alkalinity developed by large additions of lime were both injurious. The most vigorous plants were grown on the loam soil, and the differences of pH between plants grown on the limed and unlimed soil, while definite, were smaller than on the humus soil, being usually about 0.2 to 0.3 pH.

Dustman[3] used tomato plants grown in water cultures to investigate the effect of Ca. The plants were grown at pH values of 4, 5, and 6, concentrations of Ca being 1,000, 100, and 10 p.p.m. While the low-Ca

plants showed small increases in acidity of sap, it was believed that in view of the variations found in duplicate samples, the differences were not significant. Newton[15] also grew pea plants in solutions of high and low Ca content and found no increase in acidity associated with low Ca supply.

It seems probable that the internal pH of plants may sometimes be definitely changed, usually within a narrow range, by large applications of $CaCO_3$, but it has not been adequately proved that such applications are actually indispensable for the purpose of preventing an injurious lowering of the pH of the sap. To investigate this point further, plants were grown under conditions of high and low Ca supply. In order to control the supply of Ca accurately, culture solutions were used. Wheat plants were grown in solutions containing 4 p.p.m. and 100 p.p.m. of Ca. In all solutions in which the Ca concentration was decreased, the Mg concentration was also decreased with the idea of avoiding any possible complications in the relation of Ca to Mg. Determinations of pH and of buffer were made in the usual way on the expressed sap. No significant difference of initial pH was observed, but the low-Ca treatment produced an increase in the buffer against acid (fig. 4). Analyses for all the inorganic cations and anions were made.

Table 2 shows that a significant lowering of the Ca content of the sap resulted from the low-Ca treatment. The cations and anions were calculated as milliequivalents per liter, and the total of equivalents of cations compared with the total of anions as determined. Assuming that the excess cations were in equilibrium with organic-acid radicals, it is seen that, in spite of the lowering of Ca in the culture solution, and also in the sap, more bases are found in combination with organic-acid radicals in the low-Ca sap than in the high. The increase in buffer shown by the titration curve is consistent with this finding.

A different condition with respect to Ca exists in buckwheat sap. It has been assumed by other investigators that in this plant Ca is necessary to precipitate oxalic acid, which would otherwise be injurious by reason of its toxic nature. While oxalic acid may be injurious to animals, as far as the writer is aware it has not been proved that the oxalate ion, when divorced from the actual acidity produced by oxalic acid, has any toxic effect on plant growth. However, it is true that deposits of calcium oxalate are found in many plants, including buckwheat, and for this reason, oxalates are of special interest.

Buckwheat plants were grown in culture solutions containing 4 p.p.m. and 100 p.p.m. of calcium. Similar growth was made under each condition, although the yield from the low-Ca solution was about

20 per cent greater. At harvesting, the leaves and stems (including petioles) were separated, and buffer curves were obtained on the expressed saps (fig. 5). There was no change of initial pH for either the

TABLE 2

ANION AND CATION CONTENT OF SAP OF WHEAT PLANTS GROWN UNDER LOW AND HIGH-CA CONDITIONS

Culture solution	Ca, in p.p.m.	Total cations	Total inorganic anions	Excess cations
		Milliequivalents per liter		
Low Ca	75	162	19	143
High Ca	330	197	64	113

stems or leaves, but as in the case of the wheat, the buckwheat plants had a greater acid buffer in the low-Ca solution than in the high. The sap from the stems will be considered more fully. Table 3 gives a summary of the analytical results. Owing to the fact that most of the Ca in

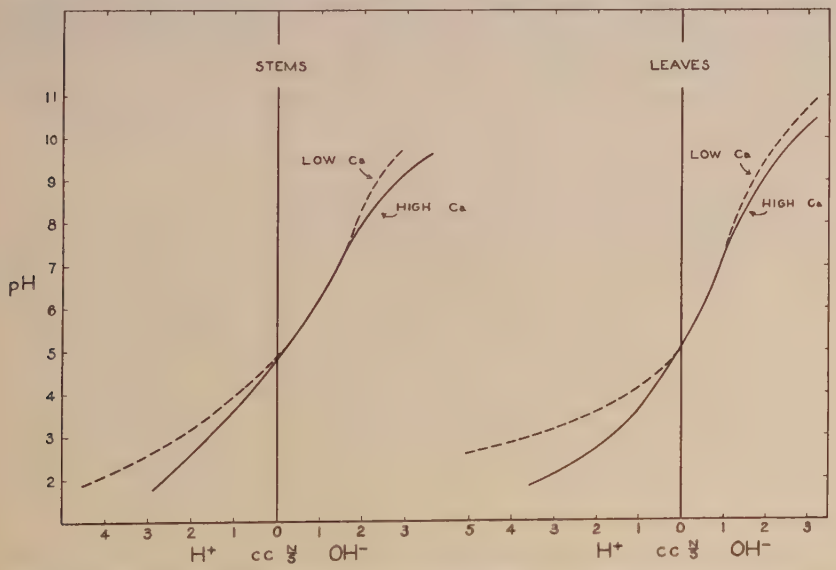

Fig. 5. Buffer curves of sap from buckwheat stems and leaves; plants grown in high and low-Ca solutions. Five cc sap was used in titrations.

buckwheat is insoluble, the concentrations are low in the sap from plants of both sets. The Ca contents of the residues left after expressing the sap were 0.20 per cent and 1.43 per cent on the dry basis, for

the low-Ca and the high-Ca plants respectively, thus proving that the low-Ca treatment was effective in reducing the calcium content of the plant as a whole. Both total equivalents of cations, and the excess of equivalents of cations over inorganic anions, were greater in the low-Ca plants (table 3). More base was available for combination with organic acids in the low-Ca plants than in the high-Ca plants, and the titration curves are consistent with this fact. The same relations were observed with the buckwheat leaves.

The decrease in Ca content of the sap was accompanied by a large increase in K. The latter then served as the main base in equilibrium with organic acids, and it is suggested that in the buffer system of the sap one base serves as well as another, provided enough total base can be absorbed. As K is readily absorbed by most plants, it is possible that the absence of Ca from the buffer system of plants of the type under discussion may have no ill effects, provided that it is present in sufficient amounts for other purposes of plant metabolism.

The changes in pH of saps as recorded in the literature have been mainly produced by applications of lime to soils. A sand-culture experiment was planned to investigate this phase of the problem. Two-gallon crocks of pure white sand were prepared and to each crock was added 1,500 cc of culture solution containing 100 p.p.m. Ca for the high-Ca set and 20 p.p.m. for the low-Ca set. In the third set $CaCO_3$ was mixed

TABLE 3

ANION AND CATION CONTENT OF SAP FROM BUCKWHEAT STEMS GROWN IN LOW AND HIGH-CA CULTURE SOLUTIONS

Culture solution	Ca, in p.p.m.	Total cations	Total inorganic anions	Excess cations
		Milliequivalents per liter		
Low Ca	59	317	182	135
High Ca	123	193	92	101

with the sand to give a content of 1.5 per cent $CaCO_3$, on the basis of dry sand. Five buckwheat plants were grown in each crock. The growth was about the same with high and low Ca supply, but the $CaCO_3$-treated plants became chlorotic. Iron tartrate was added frequently in an attempt to correct this condition, but the ultimate yield was only about one-half that of the other sets. The pH values and analytical data on

the saps are given in table 4. The titration curves are shown in figure 6. The titration for the leaves from the high-Ca solution was not made.

In both stems and leaves, the $CaCO_3$ treatment brought about an increase in pH in comparison with the high-Ca treatment. In the stems the low-Ca treatment increased the pH while in the leaves it caused a decrease. This may be explained by the fact that the sap from the stems had a higher concentration of K than was found in the sap from the

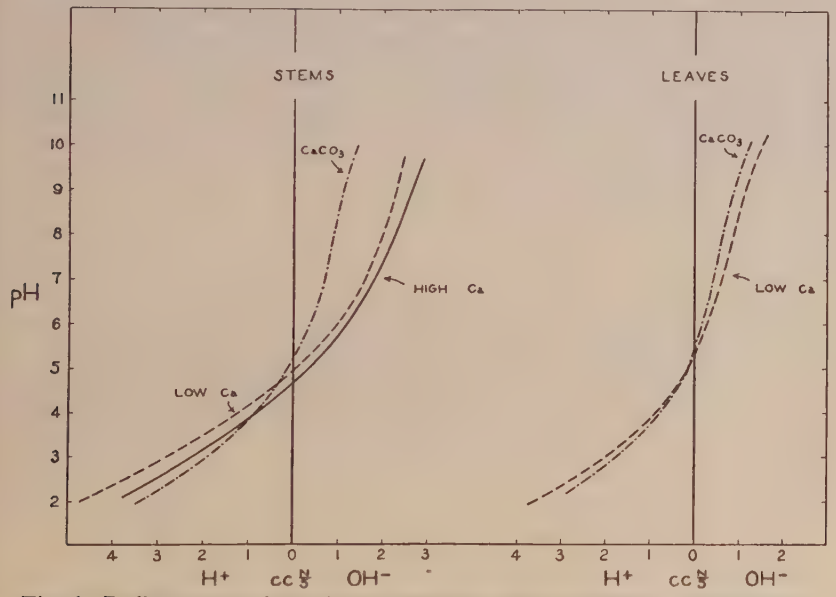

Fig. 6. Buffer curves of sap from buckwheat stems and leaves; plants grown with high Ca, low Ca, and $CaCO_3$. Five cc sap was used in titrations.

leaves, the substitution of K for Ca increasing the base content. The $CaCO_3$ treatment resulted in a large decrease in buffer on both the acid and alkaline side, which would indicate that the organic-acid, including amino acid, content was decreased. The results of analyses made for K and oxalate in the sap are given in table 4. As usual, the K values of the low-Ca plants were higher than those of the high-Ca plants. There was a slight decrease of K in the sap of the $CaCO_3$ plants. It was thought that in the $CaCO_3$ series, more K may have entered the plant as K^+ and HCO_3^- and that this would be reflected in the sap. Later evidence suggests that it is possible that even in this case more K was absorbed than by the high-Ca plants, but that a greater amount of it was precipitated.

The oxalate figures are more striking. The least amount of oxalate was present in the high-Ca set, twice as much being present in the low-Ca

set. This is probably owing to the extra equivalents of base absorbed as K in the latter set and available for combination with organic acids. In the $CaCO_3$ set, there was evidently a marked reduction in the concentration of organic-acid radicals other than oxalic. Data cited later will show that large amounts of both Ca and oxalate were precipitated

TABLE 4

pH Values and Oxalate and Potassium Content of Sap from Buckwheat Plants Grown in Sand Culture* Under Different Conditions of Ca Supply

Culture conditions	pH value		$C_2O_4^{--}$		K		Yields, fresh weight for 10 jars		
	Stems	Leaves	Stems	Leaves	Stems	Leaves	Stems	Leaves	Total
			p.p.m.		*p.p.m.*	*p.p.m.*	*grams*	*grams*	*grams*
Low Ca	4.90	5.27	2,360	.....†	9,460	5,930	480	120	600
High Ca	4.66	5.40	940	.....†	7,540	.....†	430	100	530
$CaCO_3$	5.20	5.62	3,280	.....†	7,310	5,970	230	55	285

Composition of solutions used

Solution	KNO_3	$Ca(NO_3)_2$	KH_2PO_4	$MgSO_4$	K_2SO_4	$CaCO_3$ per cent in sand	Fe, Mn, B
	mols	*mols*	*mols*	*mols*	*mols*	*per cent*	To all solutions, B was added to give approximately 0.1 p.p.m. concentration and Mn 0.3 p.p.m. Fe as 0.5 per cent solution of tartrate was added as needed to maintain green color of plants.
Low Ca	0.0075	0.0005	0.001	0.001	0.001	0.0	
High Ca	.0025	.0025	.001	.001	.002	0.0	
$CaCO_3$	0.0075	0.0000	0.001	0.001	0.001	1.5	

* Plants were grown in a greenhouse from April 29 to July 2, 1929, 2-gallon glazed earthenware crocks and pure silica sand were used.

† Analysis not made.

out of the sap, the greatest amount of both, in an insoluble form, being found in the plants from the $CaCO_3$ set. It would seem that in the plants grown in the $CaCO_3$ medium, oxalic acid was formed at the expense of other acids and that much of this oxalic acid was precipitated out, leaving a lower total acidity in the plant sap.

The results of the experiments on Ca may be briefly reviewed. If enough Ca is supplied for the maintenance of functions other than those concerned with the sap buffer system, injury to the plant may not result from a low-Ca supply, since sufficient base can be provided in the form of K. A low Ca supply usually causes an increase in organic-acid content of the sap as manifested by an increase in the buffer against acid. The change in buffer on the alkaline side is small but may show a slight decrease under conditions of low Ca supply. The actual pH of the sap

is not necessarily changed by this treatment. On the other hand, $CaCO_3$ may produce considerable increase in pH. This change seems to be definitely unfavorable to the growth of buckwheat, and possibly of many other plants. $CaCO_3$ also produces a large decrease in buffer on both sides of the neutral point in the case of buckwheat. Similar relations do not hold for melilotus.

It must be borne in mind that neither wheat nor buckwheat plants have, as a normal condition, large amounts of Ca in the sap. Buckwheat in its tissue as a whole often contains a large amount, but it is nearly all insoluble. For this reason no generalization can be made. Some plants which usually have a high Ca concentration in the sap show marked injury from a low-Ca treatment. This is the case with melilotus. The functions of Ca in plants of this type are not yet understood, but it does not appear from present evidence that the development of too great an acidity in the plant sap is the primary factor involved.

It was noted above that when the Ca supply was low there was an increased absorption of K by the plant, and a substitution in the sap of the former base by the latter. The reverse substitution may also occur, according to the results of experiments conducted in this laboratory. However, when the K supply is low, Ca, being a more slowly absorbed ion, is often not taken into the plant in sufficient quantities to permit complete substitution of bases. One result is that the pH of low-K plant sap is frequently slightly lower than that of high-K sap. The lower pH is generally accompanied by a large increase in the buffer against alkali. If the substances already suggested are responsible for the buffer in sap, this increased buffer indicates an increase in amides, amino acids, and sugars. Analytical data show this to be the case. Nightingale and coworkers[16] have also reported an increase in amide and amino nitrogen resulting from a low K supply, and many results of the same trend have been accumulated in this laboratory.

THE OXALATE SYSTEM IN BUCKWHEAT

Crystals of calcium oxalate have been observed in many plants. The high content of Ca in buckwheat plants and the fact that most of it is in an insoluble form suggests the formation of this compound. As already mentioned, it is on this basis that the rôle of Ca in this and other plants with a similar type of metabolism has been thought to be that of precipitating the oxalic acid formed.

The data presented above have proved that a decrease in the Ca supply may not be accompanied by an increase in the hydrogen-ion concentration of the expressed sap. Furthermore, according to the data presented in table 4, the oxalate content of the sap from buckwheat stems was higher with the $CaCO_3$ treatment than with the low-Ca treatment. It was therefore of interest to investigate more specifically the oxalate relations. In addition to calcium oxalate, some plant anatomists have reported crystals of potassium acid oxalate in plants. As the investigations on buffer systems had demonstrated that Ca could be more or less completely replaced by K, it was considered probable that oxalate might be converted into an insoluble form as potassium acid oxalate.

The solubility of Ca, K, and oxalate in water and in acid was investigated. Buckwheat plants were grown under controlled conditions of solution-culture technique. At harvest, the stems and leaves were separated and the plant tissues dried as quickly as possible at a temperature not exceeding 90° C. After being finely ground, different portions of the material were extracted with water, with 5 per cent HCl, and with hot 1 per cent HCl. It was observed that the constituents being investigated behaved in the same way under the last two treatments. The extraction was made with 25 parts of solvent to 1 of dry material, in an end-over-end shaker, for a period of 24 hours. The hot acid extract was made by heating for several hours on the steam bath.

The usual laboratory methods were employed for determinations of Ca and K. The following method for oxalate was developed: To the acidified aliquot to be analyzed 3 to 5 cc of 10 per cent $CaCl_2$ were added, and the solution heated. This was followed by 10 cc of 20 per cent sodium acetate. The solution was then made just alkaline to methyl red by the addition of ammonia to the boiling solution. After a few minutes' boiling, 3 cc of acetic acid (1 part acetic acid, 4 parts water) were added. This was found to bring the solution to pH 5.0–5.2. The solution was filtered, preferably after standing, and washed well to remove soluble calcium. The precipitate containing calcium oxalate plus some organic matter was dissolved in HCl. The solution was then evaporated to dryness and the residue ignited. The Ca content of the ash was determined and from it the oxalate present in the sample calculated.

The results from analyses on buckwheat leaves grown under culture conditions of low Ca, high Ca, and $CaCO_3$ are given in table 5. This set is similar to one discussed previously in connection with hydrogen-ion concentration and buffer titration. In columns 1 to 6 are given the amounts of each ion found in the extracts. In column 9 are given

the equivalents of water-insoluble oxalate minus water-insoluble Ca. On the assumption that the oxalate thus computed existed as KHC_2O_4, the equivalents of $HC_2O_4^-$ would be only half the number expressed as $C_2O_4^{--}$. On this basis there is a suggestive agreement between the amounts of insoluble K and of residual oxalate.

TABLE 5

Ca^{++}, K^+, AND $C_2O_4^{--}$ DISSOLVED FROM BUCKWHEAT STEMS BY DIFFERENT SOLVENTS

Culture conditions	Milliequivalents of certain ions for 100 grams dry weight										
	Soluble in H_2O			Soluble in HCl*			Insoluble in water				
	$C_2O_4^{--}$	Ca^{++}	K^+	$C_2O_4^{--}$	Ca^{++}	K^+	$C_2O_4^{--}$	Ca^{++}	$C_2O_4^{--}$—Ca^{++}	K^+	$^*HC_2O_4^-$†
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*
In leaves											
Low Ca	23	—‡	35	128	75	46	105	75	30	11	15.0
High Ca	20	—‡	30	120	86	38	100	86	14	8	7.0
$CaCO_3$	31	—‡	34	158	110	46	127	110	17	12	8.5
In stems											
Low Ca	20	1	114	62	34	125	42	33	.....	11	.....
High Ca	13	2	71	62	46	102	49	44	.....	31	.....
$CaCO_3$	15	4	94	89	104	114	74	100	.....	20	.....

* Cold 5 per cent acid was used for the stems and hot 1 per cent acid for the leaves.

† On the assumption that the oxalate computed by subtracting insoluble Ca^{++} from insoluble $C_2O_4^{--}$ (col. 9) existed as KHC_2O_4, the $HC_2O_4^-$ would be only half this oxalate.

‡ Amount negligible for present purpose.

The inference can reasonably be drawn that in buckwheat leaves oxalic acid formed in metabolism may be precipitated by either Ca or K. This is contrary to the contention that Ca in the form of $CaCO_3$ is necessary for the precipitation of oxalic acid. It is also evident that not only is the total oxalate concentration highest in the $CaCO_3$-treated plants, but that the water-soluble oxalate is also highest in this set.

Analyses were also made on the stems of this set, the acid extract being made with cold 5 per cent HCl. The material was shaken for 24 hours, simultaneously with the water extract. The acid extract of the stems had a higher K and lower Ca content than that of the leaves and in this case also large amounts of both elements were insoluble in water (table 5). There are more than enough equivalents of Ca and K in an insoluble form to account for the oxalate precipitated. Insoluble compounds of these elements other than oxalates may be formed. The fig-

ures for the $CaCO_3$ set show that this is undoubtedly the case for Ca, for even if the insoluble K is not included, there is still an excess of insoluble Ca over insoluble oxalate.

As before, the total oxalate was highest in the plants receiving the $CaCO_3$ treatment. It would seem that the excess oxalate was formed in response to the presence of $CaCO_3$ in the medium, rather than that the Ca was essential to precipitate the oxalic acid necessarily formed as a result of metabolic processes.

It is of interest that in this experiment slightly more growth was made by the low-Ca plants than by the high-Ca plants. The plants grown in the medium containing $CaCO_3$ had only about half the weight of those grown in the other media (table 4). In both stems and leaves the concentration of water-soluble oxalate was higher in the low-Ca set than in the high, and in the stems it is highest of all in the low-Ca plants. This seems to refute the idea that oxalate is injurious to growth of this type of plant. In both stems and leaves, water-soluble K is highest in the low-Ca plants. It is probable, therefore, that as long as sufficient base is present, an increase in oxalate is not injurious.

It might be argued that the extra amount of K found in the water extract of the plant tissues was responsible for the increased growth, regardless of the oxalate concentration. It will be observed, however, that both water-soluble and total K are higher in the $CaCO_3$ plants than in the high-Ca plants, and yet no increase in growth resulted. This is not conclusive, for the $CaCO_3$ may have counteracted the beneficial effect of the K absorbed. As suggested above, it is possible that the additional K was absorbed in this case as K^+ and HCO_3^-, and that this was in part responsible for the alkalinity observed in the expressed sap of the $CaCO_3$ plants.

Another experiment was conducted with culture solutions, using three solutions: (1) high Ca and K, (2) low Ca, and (3) low K. The dried material was extracted with water and 5 per cent HCl. The analyses are given in table 6. In the low-K set all the potassium is in a water-soluble form. There is more insoluble Ca than insoluble oxalate, indicating that some Ca exists in other insoluble forms. In the low-Ca plants, there is a comparatively small amount of insoluble oxalate present. In this case the equivalents of insoluble K alone far exceed those of insoluble oxalate, suggesting that some K may go out of solution in some form other than KHC_2O_4. It may be that precipitation as $KH_3(C_2O_4)_2$ occurs in some cases, which would make the discrepancy still greater. Again in this set, the highest total oxalate concentration was associated with the highest Ca content.

In the low-Ca plants, at the pH of the sap, the soluble oxalate would be present, if in equilibrium with K, as $K_2C_2O_4$. Assuming this equilibrium to exist, there would not be enough water-soluble K to form salts with the oxalate ions present. As the Mg was probably very low, being supplied in small amount, it is possible that there was an accumulation of oxalic acid with lowering of pH. The fact that in this experiment the yield from the low-Ca set was less than the high-Ca set, strengthens this assumption.

TABLE 6

Ca^{++}, K^+, AND $C_2O_4^{--}$ DISSOLVED FROM BUCKWHEAT STEMS BY DIFFERENT SOLVENTS

Culture conditions*	Milliequivalents of certain ions for 100 grams dry weight									Yields, dry weight, for 36 jars		
	Soluble in H_2O			Soluble in HCl†			Insoluble in H_2O					
	$C_2O_4^{--}$	Ca^{++}	K^+	$C_2O_4^{--}$	Ca^{++}	K^+	$C_2O_4^{--}$	Ca^{++}	K^+	Stems	Leaves	Total
										grams	*grams*	*grams*
Low Ca	62	—‡	41	81	21	94	19	21	53	525	185	710
High K	4	5	34	111	104	73	107	99	39	695	250	945
Low K	5	46	9	159	232	9	154	186	0	480	240	720

Composition of culture solutions used

Solution	KNO_3	$Ca(NO_3)_2$	KH_2PO_4	$MgSO_4$	K_2SO_4	Fe, Mn, B
	mols	*mols*	*mols*	*mols*	*mols*	
Low Ca	0.005	0.0002	0.0004	0.0004	0.0006	Treatment similar
High K	.005	.0050	.0004	.001	.0000	to that described
Low K	0.000	0.0050	0.0004	0.001	0.0000	in table 4

* Plants were grown in a greenhouse from June 28 to July 22, 1929; 2-liter jars were used with 2 plants in each jar.

† Cold, 5 per cent acid.

‡ Amount negligible.

The theory that absorption of Ca is necessary in order to precipitate oxalic acid formed in plant metabolism, is not substantiated. It would appear to be immaterial whether the oxalate is in a soluble or insoluble form, provided sufficient base, either Ca or K, is present. Furthermore, it is shown that when the Ca content of buckwheat plants is markedly increased by growing them either in the presence of an excess of $CaCO_3$, or in a low-K solution, there is a decided increase in the amount of oxalate formed. An increased yield of oxalate may possibly result from an upsetting of metabolism caused by these treatments. On the other hand, plants grown in a solution low in Ca do not necessarily show a similar increase of oxalate.

SUMMARY

In the light of previous investigations conducted by the author, a further attempt was made to identify the principal types of substances responsible for the buffer of plant saps. All work was done on sap expressed from tissues frozen and thawed. It is concluded that organic acids, amides, amino acids, phosphates, and sugars are the substances of most importance. The data suggest that in studies on plant metabolism, titration curves may be a useful means of ascertaining large changes in some of the organic constituents of the sap.

A low phosphate supply resulted in an increase of hydrogen–ion concentration and buffer in the sap. This probably indicates an increase in organic acids, amides, amino acids, and possibly sugars. Similar changes may occur as a result of low K supply.

The importance of Ca in the plant buffer system was studied. It is shown that Ca is not an indispensable part of the buffer system of the plants studied. Such plants when grown with a low Ca supply do not necessarily show an increase of hydrogen-ion concentration in the sap, nor are they always injured. Under these conditions, the base necessary for the buffer system is supplied by an increased absorption of K. On the other hand, $CaCO_3$ treatment may produce a condition of alkalinity which is definitely injurious to some plants. Furthermore, $CaCO_3$ was not found to cause a decrease in the oxalate content of plant sap in buckwheat.

The oxalate system in buckwheat was investigated. A large proportion of the oxalate is usually in an insoluble form. The data indicate that this may be precipitated either with Ca or K. The theory that $CaCO_3$ or $Ca(HCO_3)_2$ is necessary for the neutralization of organic acids in such plants is not substantiated. The high Ca content undoubtedly indispensable for good growth of certain types of plants seems to require some other explanation.

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